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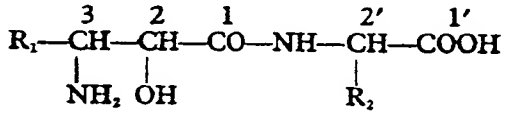
(54) IMPROVEMENTS IN AND RELATING TO BESTATIN DERIVATIVES

(71) We, ZAIDAN HOJIN BISEIBUTSU KAGAKU KENKYU KAI, a Japanese Company of 3—15—23 Kamiosaki, Shinagawa-ku, Tokyo, Japan do hereby declare the invention for which we pray that a patent may be granted to us and the method by which it is to be performed to be particularly described in and by the following statement:—

The invention relates to a derivative of bestatin. Derivatives of bestatin having the general formula:



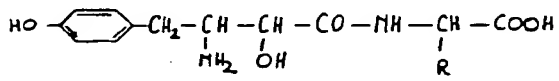
(I)

in which R_1 is a lower alkyl group, cycloalkanolalkyl group, phenyl group, benzyl group and/or a substituted benzyl group; R_2 is an alkyl group having 1 to 6 carbon atoms, hydroxyalkyl group, mercaptoalkyl group, carboxamidoalkyl group, alkoxyalkyl group alkylmercaptoalkyl group, carboxyalkyl group, aryl group, aralkyl group or substituted aralkyl group are generally known. Bestatin itself is (2S,3R) - 3 - amino - 2-hydroxy - 4 - phenylbutanoyl - S - leucine and is well known for enhancing the anti-tumour effect of Bleomycin.

The present Applicants have found that compounds having the general formula (I) above enhance the properties when a *p*-hydroxybenzyl group is substituted for R_1.

According to the present invention, therefore, there is provided a compound having the general formula:

Formula A



in which R is an alkyl group having 1 to 6 carbon atoms, hydroxyalkyl group, mercaptoalkyl group, carboxamidoalkyl group, alkoxyalkyl group, alkylmercaptoalkyl group, carboxyalkyl group, an aryl group, an aralkyl group or a substituted aralkyl group.

In a particular embodiment of the present invention R is an isobutyl group, but it will be appreciated that, as stated above R may be any substituent stated. In which case the substitution of R into the Bestatin molecule is expected by known means. The invention is particularly concerned with (2RS,3RS) - 3 - amino - 2 - hydroxy - 4-p - hydroxyphenylbutanoyl - (S) - leucine (A) and to (2S,3R) - 3 - amino 2 - hydroxy - 4 - *p* - hydroxy - phenylbutanoyl - (S) - leucine (B).

The particular compounds referred to above hereinafter referred to as Examples A and B have shown to have enhanced physiological activities as follows:—

(A) Inhibitory activity against aminopeptidase B Testing method:

The method described by V. K. Hopusu, K. K. Makinen, G. G. Glenner in Archives of Biochemistry and Biophysics, *114*, 557, (1966) was modified. To the mixture of 0.3 ml of 1 mM arginine β-naphthylamide and 1.0 ml of 0.1 M Tris hydrochloride buffer (pH 7.0), 0.7 ml of distilled water with or without a test material is added and warmed at 37°C for 3 minutes. The reaction is started by addition of

0.2 ml of aminopeptidase B solution which is prepared by Sephadex (Registered Trade Mark) 100 chromatography as described by Hopusu et al. After 30 minutes at 37°C, 0.6 ml of 1.0 M acetate buffer (pH 4.2) containing diazonium salt of *o*-amino-azotoluene at 1.0 mg/ml and Tween (Registered Trade Mark) 20 at 1.0% is added. Fifteen minutes at room temperature thereafter, absorbance (a) at 530 nm is measured by spectrophotometer. As the control, by similar means, the absorbance (b) after the reaction in the absence of a sample is measured. The inhibition percent is calculated as follows: $(b-a)/b \times 100$.

Inhibition percentages at various concentrations were measured and, from the measured inhibition percentages, 50% inhibitions (ID_{50}) were deduced. The results are as listed in the Table 1.

TABLE 1

Compounds	ID_{50} (μg/ml)
Example A	0.10
Example B	0.007
Bestatin [(2S,3R)-AHPA-(S)-Leu]	0.10

AHPA 3 - amino - 2 - hydroxy - 4 - phenylbutanoic acid residue; Leu; leucine.

As will be seen from Table 1, the compound of Example A has substantially the same inhibitory effects as Bestatin but the compounds of Example B can attain the same effect in a far less amount, one-fourteenth of Bestatin. Gathering from these results, it is expected that the new compounds, especially the compound of Example B which is an optically active form of the compound of Example A, can be an extremely useful physiologically active substance.

The compound of Example A was also tested for its humoral antibody formation to find its efficacy as an immunizing cancer inhibitor. As a result, it was found that the compound has an effect of increasing the number of humoral antibody cells to a considerable degree, as shown in Experimental Example which will appear hereinlater. The results suggest that the compound can serve as an excellent immunizing cancer inhibitor. For the humoral antibody formation of Bestatin *per se* please refer to "The Journal of Antibiotics" Vol. 29 No. 8 pp 857—859, August 1976.

Following is a description by way of example only of methods of carrying the invention into effect:

EXAMPLE A

Step 1

In the following Example the following abbreviations are used; Z; benzyloxy-carbonyl residue; AHPA(p-OH); 2 - hydroxy - 4 - p - hydroxyphenylbutanoic acid residue; AHPA(p-OZ); 3 - amino - 2 - hydroxy - 4 - p - benzyloxycarbonyloxy - butanoic acid residue; HOBt; 17 - hydroxybenzotriazole; Leu - OB_3l; ToSOH; L - leucine-benzyl ester, toluenesulfonic acid salt, DCCD; dicyclohexylcarbodiimide and DCHA; dicyclohexylamine.

22.0 g of oily Z - (2RS,3RS) - 3 - amino - 2 - hydroxy - 4 - (p' - methoxy-phenyl)butyronitrile is dissolved in a mixture of 200 ml of conc. hydrochloric acid and 200 ml of dioxane. After adding 13.2 g of anisole, the reaction mixture is refluxed for 12 hours. Then dioxane is distilled away under reduced pressure, the concentrated solution is washed with ether and the aqueous layer is concentrated under reduced pressure and evaporated to dryness. Subsequently, 100 ml of water is added to the residual substance and the insoluble substance is separated by filtration. After adding an equal quantity of acetone, the mixture is adjusted to pH 5.5 with ammonia water. The mixture is allowed to stand in a refrigerator. The deposited crystals are separated by filtration, 6.73 g of intended (2RS,3RS)-AHPA (*p*-OH) is obtained.

Step 2

2.11 g of (2RS,3RS)-AHPA-(*p*-OH) obtained in Step 1 is dissolved in 10 ml of 1N sodium hydroxide solution. While vigorously agitating the solution under cooling with ice, 4.5 ml of Z-Cl is added in three portions over a period of 30 min. More than the reaction mixture is vigorously agitated for 1 hour under cooling with ice and for 3 hours at room temperature. During the reaction pH is adjusted to 8—9 with 1N sodium hydroxide solution.

When the reaction has been completed, 6N hydrochloric acid is added to adjust the reaction mixture to pH 2. As a result, oily material is separated which is then extracted twice with 100 ml of ethyl acetate. The ethyl acetate layer is washed with water and dehydrated to dryness by use of anhydrous magnesium sulfate. After separating magnesium sulfate by filtration, the filtrate is concentrated under reduced

pressure and the residue is crystallized in ethyl acetate-petroleum ether to prepare 3.64 g of Z - (2RS,3RS) - AHPA(*p* - OZ).

m.p. 138—140°C.

Step 3

4.79 mg of Z - (2RS,3RS) - AHPA(*p* - OZ) and 162 mg of HOBt are dissolved in 10 ml of tetrahydrofuran. After adding 4.72 mg of Leu - OBzl . TosOH, the mixture is neutralized with 0.168 ml of triethylamine and cooled to —5°C. Then 206 mg of DCCD is added and the reaction mixture is allowed to stand overnight for reaction. Tetrahydrofuran is distilled away under reduced pressure and 30 ml of ethyl acetate is added. After filtering off the insoluble substances, the filtrate is washed with 1N sulfuric acid, water, 5% aqueous sodium bicarbonate solution and water in this order and then dehydrated to dryness with anhydrous magnesium sulfate. The residue obtained by concentrating the filtrate under reduced pressure is solidified in ethyl acetate-petroleum ether. Recrystallization from the same solvent gives 450 mg of Z - (2RS,3RS) - AHPA(*p* - OZ) - (S) - Leu - OBzl.

m.p. 98—99°C, $[\alpha]_{578}^{18}$ —14.0° (c 0.58, AcOH).

Step 4

400 mg of Z - (2RS,3RS) - AHPA(*p* - OZ) - (S) - Leu - OBzl is dissolved in 10 ml of methanol and hydrogenated for 3 hours with about 10 mg of palladium black. The catalyst is filtered off and the solvent is concentrated under reduced pressure. When a recrystallization operation is carried out with methanol - ethyl acetate, 219 mg of (2RS,3RS) - 3 - amino - 2 - hydroxy - 4 - *p* - hydroxyphenylbutanoyl - (S)-leucine is obtained.

$[\alpha]_{578}^{30}$ —8.8° (c 0.90, AcOH),
Rf 0.48 and 0.51,
Anal. for $C_{16}H_{24}N_2O_6$,

Found:	C 59.38,	H 7.23,	N 8.95
Calculated.:	C 59.24,	H 7.46,	N 8.64

Example B

When 30 g of Z - (2RS,3R) - 3 - amino - 2 - hydroxy - 4 - *p* - hydroxyphenylbutyronitrile is treated in the similar manner to Example A, Step 1, 12.61 g of (2RS,3R) - AHPA(*p* - OH) is obtained.

Rf 0.20,
Anal. for $C_{10}H_{12}NO_4$,

Found:	C 58.63,	H 5.99,	N 7.43
Calculated:	C 58.81,	H 5.92,	N 7.82

When (2RS,3R) - AHPA(p - OH) is benzyloxycarbonylated using benzyl - S-4,6 - dimethylpyrimidine - 2 - ylthiolcarbonate, Z - AHPA(p - OH) is obtained as DCHA salt.

15.22 g of crude DCHA salt was crystallized from methanol, ethyl acetate and petroleum ether, and 3.2 g of optically impure Z - (2R,3R) - AHPA)p - OH) DCHA salt is obtained as a first crop.

When the mother liquor is evaporated to dryness, and the residue is precipitated three times from ethyl acetate and ether, 5.02 g of optically pure Z - (2S,3R) - AHPA-(p - OH)DCHA salt is obtained.

m.p. 121—122°C, $[\alpha]_{578}^{20}$ +49.9° (c 0.87, AcOH),
Anal. for $C_{30}H_{42}N_2O_6$,

Found:	C 69.81,	H 8.35,	N 6.42
Calculated:	C 69.46,	H 8.16,	N 6.17

After a treatment of Z - (2S,3R) - AHPA(p - OH) DCHA salt (1.05 g) with ethyl acetate and dil. H_2SO_4, the obtained Z - (2S,3R) - AHPA(p-OH), 866 mg of (S) - Leu - OBzl . TosOH, 405 mg of HOBt, 0.308 ml of triethylamine and 412 mg of DCCD are treated in the similar manner to Example A, Step 3. Oily Z - (2S,3R)-AHPA(p - OH) - (S) - Leu - OBzl is obtained quantitatively.

When the obtained oily Z - (2S,3R) - AHPA(p - OH) - (S) - Leu - OBzl is treated

in the similar manner to Example A, Step 4, 630 mg of (2S,3R) - 3 - amino - 2-hydroxy - 4 - *p* - hydroxyphenylbutanol - (S) - leucine is obtained,

$[\alpha]_{578}^{29}$ −19.9° (c 1.19, AcOH),
Rf 0.48,
Anal. for $C_{16}H_{28}N_2O_6$,

Found:	C 59.98,	H 7.42,	N 10.42
Calculated:	C 60.55,	H 7.30,	N 10.08

Experimental Example 1

Effect of (2RS,RS) - 3 - amino - 2 - hydroxy - 4 - hydroxyphenylbutanoyl-(S) - leucine on humoral antibody formation to Sheep Red Blood Cell (SRBC) in mice was studied as follows. Mice (dd/Y female) were immunized by intravenous injection of 10^8 SRBC. Intraperitoneal injection of (2RS,RS) - 3 - amino - 2 - hydroxy - 4 - *p*-hydroxyphenylbutanoyl - (S) - leucine was made soon afterwards.

Bestatin [(2S,3R) - 3 - amino - 2 - hydroxy - 4 - phenyl - butanoyl - (S)-leucine] and (2RS,3RS) - 3 - amino - 2 - hydroxy - 4 - *p* - chlorophenylbutanoyl-(S) - leucine were used as control:

Four days thereafter, the number of plaque forming cell in spleen were enumerated by "Jerne's" hemolytic plaque technique (Jerne, N. K.; A. A. Nordin & C. Henry: "The agar plaque technique for recognizing antibody-producing cells" in cell-bound antibodies (Wistar Institute Press Philadelphia (1963), pp. 109—122).

The results are as listed in Table 1.

TABLE 2

Effect of (2RS,RS) - 3 - amino - 2 - hydroxy - 4 - hydroxyphenylbutanoyl-(S) - Leucine on humoral antibody formation to SRBC in mice

Name of compound	Dose	Antibody forming cell treated group Number	non-treated group
—	—	12500 ± 9050	—
(2RS,3RS) - 3 - amino-2 - hydroxy - 4 - *p*-hydroxyphenylbutanoyl - (S) - leucine	1 mg	207400 ± 8025	1.66
	100 μg	261000 ± 11700	2.09
	10 μg	208000 ± 7180	1.66
	1 μg	175800 ± 8200	1.41
	0.1 μg	141000 ± 5700	1.13
Bestatin	1 mg	190000 ± 7100	1.52
	10 μg	136250 ± 6500	1.09
(2RS,3RS) - 3 - amino-2 - hydroxy - 4 - *p*-chlorophenylbutanoyl - (S)-leucine	1 mg	208750 ± 8000	1.67
	10 μg	133750 ± 5600	1.07

The number of antibody forming cells in mice group given 10 μg of Bestatin or (2RS,3RS) - 3 - amino - 2 - hydroxy - 4 - *p* - chlorophenylbutanoyl - (S) - leucine was nearly equal to that of non-treated group.

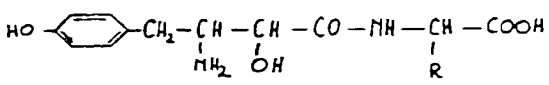
On the other hand the number of antibody forming cells in mice of group given 10 μg of (2RS,3RS) - 3 - amino - 2 - hydroxy - 4 - *p* - hydroxyphenylbutanoyl - (S)-leucine was 1.66 times larger than that of non-treated group, and even when given only 1 μg it showed 1.41 times.

As mentioned above, (2RS,3RS) - 3 - amino - 2 - hydroxy - 4 - *p* - hydroxybutanoyl - (S) - leucine has excellent effect of increased number of antibody forming cells.

Furthermore the tested compounds did not increase the weight of the spleen or the number of nonspecific antibody forming cells.

WHAT WE CLAIM IS:—

1. A compound having the general formula A



in which R is an alkyl group having 1 to 6 carbon atoms, a hydroxyalkyl group, a mercaptoalkyl group, a carboxamidoalkyl group, alkoxyalkyl group, alkylmercaptoalkyl group, carboxyalkyl group, aryl group or a substituted aralkyl group.

2. (2RS,3RS) - 3 - amino - 2 - hydroxy - 4 - *p* - hydroxy - phenylbutanoyl-(S) - leucine.

3. (2S,3R) - 3 - amino - 2 - hydroxy - 4 - *p* - hydroxy - phenylbutanoyl - (S)-leucine.

4. A method of preparing compounds as claimed in claim 1 substantially as described in either of the specific Examples herein set forth.

5. A pharmaceutical composition comprising a compound of the general formula A wherein R has the meanings given above and a pharmaceutically acceptable carrier or diluent therefor.

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Erfinder: UMEZAWA HAMAO
TAKEUCHI TOMIO
AOYANAGI TAKAAKI
ISHIZUKA MASAAKI

Anmelder: MICROBIAL CHEM RES FOUND

Titel: IMMUNO-CARCINOSTATIC AGENT

Zusammenfassung

PURPOSE: Pharmaceutials comprising bestatins which improve the immunity, inhibit the metastatis of cancer and prevent the relapse of cancer, and other carcinostatic agents in option.